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Limited Polymorphism of the *Plasmodium vivax* Merozoite Surface Protein 1 Gene in Isolates from Turkey

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Abstract. The 200-kD merozoite surface protein of *Plasmodium vivax* (PvMSP-1) is one of the leading vaccine candidates against *P. vivax* malaria. However, the gene encoding PvMSP-1 (*pvmsp1*) is highly polymorphic and is a major obstacle to effective vaccine development. To further understand polymorphism in *pvmsp1*, we obtained 30 full-length *pvmsp1* sequences from southeastern Turkey. Comparative analysis of sequences from Turkey and other areas showed substantially limited polymorphism. Substitutions were found at 280 and 162 amino acid sites in samples from other regions and those from Turkey, respectively. Eight substitutions were unique to Turkey. In one of them, D/E at position 1706 in the C-terminal 19-kD region, the K/E change at 1709 was the only polymorphism previously known. Limited diversity was also observed in microsatellites. Data suggest a recent population bottleneck in Turkey that may have obscured a signature for balancing selection in the C-terminal 42-kD region, which was otherwise detectable in other areas.

INTRODUCTION

Malaria imposes a huge public health burden in tropical and subtropical countries with one million deaths every year.[1] Of the five human malaria parasites, *Plasmodium vivax* is the most prevalent in Asia, Melanesia, the Middle East, South and Central America, accounting for 70–80 million cases annually.[2] Although *P. vivax* is often regarded as a benign and self-limiting infection, it can lead to debilitating illness and remains a major cause of morbidity in malaria-endemic countries. Emergence of drug-resistant *P. vivax* makes the control of *P. vivax* malaria more difficult.[3] Thus, there is an increasing demand for developing effective vaccines against *P. vivax* malaria with potential targets directed against the asexual blood stages, which are responsible for clinical manifestations of the disease.[4] The 200-kD merozoite surface protein 1 (MSP-1), which is abundantly expressed on the surface of merozoites, is one of the leading asexual blood stage vaccine candidates.[5] MSP-1 is conserved in all *Plasmodium* species[6] and is essential for parasite survival.[7]

Plasmodium falciparum MSP-1 undergoes proteolytic processing, producing four major polypeptides of 83-kD, 30-kD, 38-kD, and 42-kD.[5] Coincident with erythrocyte invasion, the C-terminal 42-kD protein is further cleaved to produce the N-terminal 33-kD and C-terminal 19-kD fragments, with all, except one, processed fragments shed.[8] The C-terminal 19-kD fragment, which contains cysteine-rich epidermal growth factor–like domains, remains anchored to the merozoite membrane and is carried into the invaded erythrocytes. Both the 42-kD and 19-kD polypeptides are considered to be promising vaccine candidates for *P. falciparum* and *P. vivax*.[4,9] However, undoubtedly, the gene encoding MSP-1 (*msp1*) is highly polymorphic[6,10,11] and, thus, presents a major obstacle to effective vaccine development. *Plasmodium vivax msp1* (*pvmsp1*) shows extensive allelic variation and is subject to balancing selection,[6,12] suggesting an involvement of parasite evasion from host immune attack.

According to inter-allelic sequence variation, *pvmsp1* contains six highly polymorphic regions interspersed with conserved blocks.[13] Of note is the limited polymorphism in the C-terminal 19-kD in *pvmsp1* with only one amino acid substitution, K/E at 1709,[14] in contrast to five major amino acid substitutions in *P. falciparum msp1*.[11] With its polymorphic nature, *pvmsp1*, particularly the highly polymorphic poly Q region in block 6,[13] previously referred to as conserved block (CB) 5,[15] has frequently been used as a molecular marker to monitor genetic diversity of *P. vivax* in different populations.[16–23] However, investigations on polymorphism of the whole *pvmsp1* have until now been limited.[13]

In this study, we report polymorphism of *pvmsp1* from isolates in Sanliurfa, southeastern Turkey, where malaria has long been one of the most common infectious diseases and *P. vivax* has continuously been identified as the only *Plasmodium* species. Our previous study has identified a high prevalence of persons with naturally acquired antibodies to PvMSP1 in southeastern Turkey.[24] We obtained 30 full-length *pvmsp1* sequences from the same study area. Comparative analysis of polymorphism in *P. vivax* populations from Sanliurfa and other areas showed substantially limited *pvmsp1* polymorphism in parasite populations in Turkey with some polymorphism unique to this country.

MATERIALS AND METHODS

Parasite isolates and DNA extraction. *Plasmodium vivax* isolates were obtained in two towns, Siverek and Harran, in Sanliurfa Province, southeastern Turkey (Figure 1), where malaria persists throughout the year with high rates during July–November.[24] According to the World Health Organization, in 2006 *P. vivax* transmission was reported in seven provinces of Turkey and 84% of the cases were from Diyarbakir and Sanliurfa.[1] In the past decade, malaria incidence rapidly decreased in this area because of government malaria control efforts that used chloroquine and primaquine.[1] Turkey shows a strong political commitment to the Tashkent Declaration, endorsed in 2005, and malaria surveillance activities have

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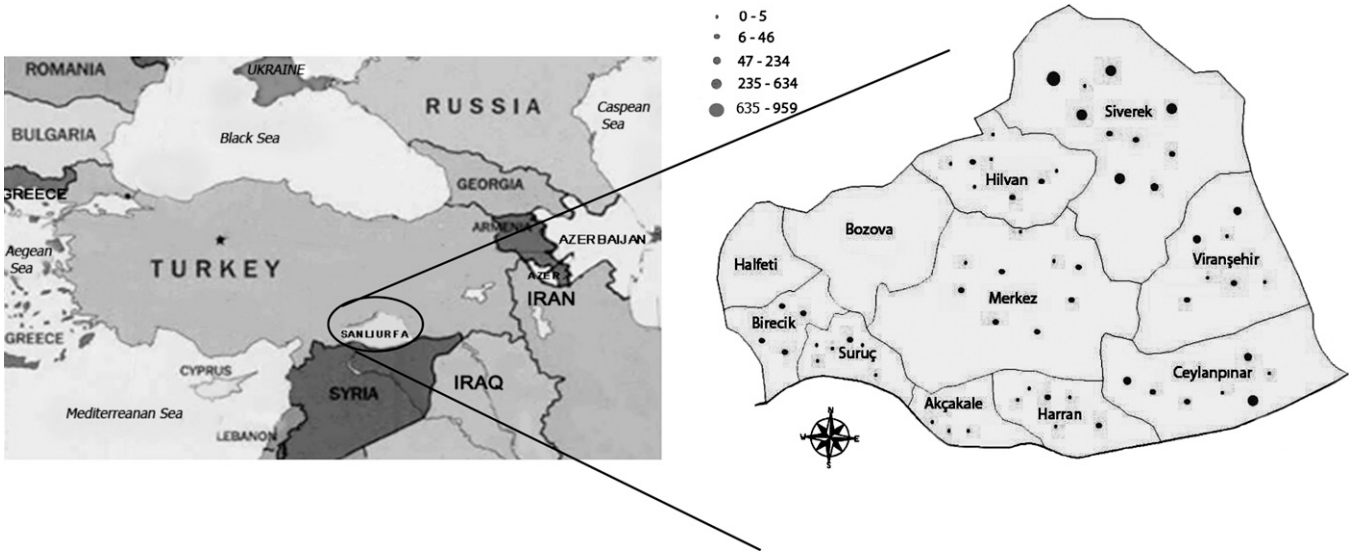

FIGURE 1. Map of Turkey showing Sanliurfa, the study area, where *Plasmodium vivax* isolates were obtained in Siverek and Harran provinces. Circles on the right indicate the number of malaria cases each year during 2001–2009 in provinces in Sanliurfa.

been intensified throughout the country, and priority has been given to provinces in southeastern Anatolia.

A total of 31 blood samples were obtained from patients diagnosed microscopically with *P. vivax* infection at several National Malaria Control Centers within Siverek and Harran: 20 from Siverek during July–December in 2007 and 11 from Harran during September–November in 2008. Although these samples were limited in number (n = 31), they include 32% of total *P. vivax* cases (n = 96) in the study area: 47 in 2007 and 49 in 2008 (Figure 2). Mean age of patients was 21 years (range = 2–55 years) and 61% were male. Giemsa-stained thick blood smears were used to calculate parasitemia (parasites/microliter of blood) as described.[25] Mean ± SD parasite density was 5,057 ± 757 parasites/μL (range = 480–14,720 parasites/μL). The patients were selected randomly from different ethnic and racial groups. All samples were collected after informed consent was obtained from patients or their parents. Sampling authorization was obtained from the Turkish Ministry of Health Sanliurfa Bureau, and ethical approval was obtained from the Research Institute for Microbial Diseases, Osaka University.

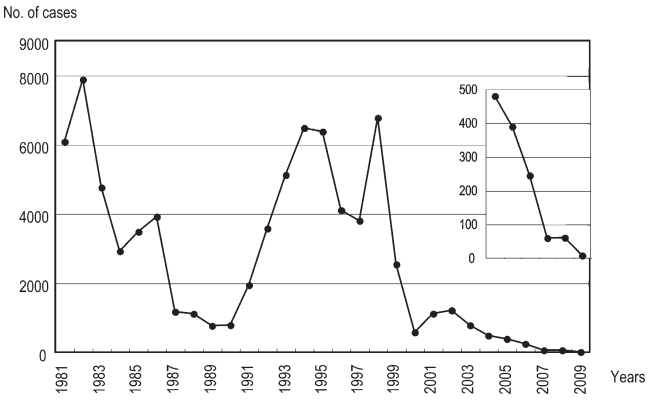

FIGURE 2. Annual incidence of *Plasmodium vivax* cases in Sanliurfa, southeastern Turkey, according to the National Malaria Control Center in Sanliurfa during 1981–2009. The y-axis is scaled for 2004–2009 to show the incidence (**inset** on the right).

An aliquot of venous blood (100–200 μL) was taken by finger prick, spotted onto Whatman® 31ETCHR filter paper (Whatman, Piscataway, NJ), and air-dried. DNA was extracted from filter blots using the EZ1 BioRobot™ (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Sequencing. Full-length *pvmsp1* (5.2–5.3 kb) was amplified by polymerase chain reaction (PCR) using Takara LA Taq (Takara Bio, Otsu, Japan) in a 20-μL reaction mixture as described[26] with primers PVF0 and PVR0.[13] Forty cycles of amplification (20 seconds at 93°C and 5 minutes at 62°C) were preceded by denaturation at 93°C for 1 minute and followed by final elongation at 72°C for 10 minutes. The PCR product was diluted 10-fold, and a 2-μL aliquot was used as template for a second PCR amplification of 20 cycles in a 50-μL reaction mixture using primers PVF0-2 (5′-CGTACATCTTTAAACCCCACACACT-3′) and PVR0. The PCR products were purified by using QIAquick (PCR Product Purification kit; Qiagen). DNA sequencing was performed directly from two independent PCR products, using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI 3130 Genetic Analyzer (Applied Biosystems). Sequencing primers were designed to cover specific regions in both directions as described.[13] Four microsatellite loci(MS8, MS9, MS15, and 3.502)[27,28] were also sequenced after PCR amplification by using specific primers shown in Supplementary Table 1. The PCR conditions were the same as for *pvmsp1* and a second amplification was not performed. Contiguous sequences were constructed by using ATGC version 4.01 (Genetyx Corp., Tokyo, Japan). Mixed genotype infections judged from overlapping peak at given positions in an electrophelogram were excluded from further analysis. Sequences obtained in this study have been deposited to DNA Database of Japan/European Molecular Biology/GenBank under accession numbers AB564559–AB564588.

Statistical analysis. The *pvmsp1* sequences obtained in this study were analyzed with previously published full-length

sequences (n = 43) from Thailand (n = 20), Brazil (n = 9), South Korea (n = 4), India (n = 1), El Salvador (n = 1), Bangladesh (n = 5), Vanuatu (n = 2), and Côte d'Ivoire (n = 1) (GenBank accession numbers AF435593–AF435599, AF435601–AF435620, AF435622–AF435625, AF435627, AF435629–AF435632, AF435634–AF435639, and DQ220742) (http://www.ncbi.nlm.nih.gov/Genbank/index.html). Between-population comparison of full-length *pvmsp1* was made by using samples from Turkey, Thailand and Brazil. Isolates from Thailand were obtained from Tak Province in 1997–1998, and isolates from Brazil were obtained in Rondonia in 1995 and 1997.[13] Partial sequences of the C-terminal 42-kD region from isolates from India (n = 28)[29] were obtained from GenBank. Partial *pvmsp1* sequences were also obtained from Azerbaijan (n = 36),[19] Iran (n = 191),[21,22,30] Afghanistan (n = 57),[23] Pakistan (n = 33),[30] Myanmar (n = 135),[31] Thailand (n = 33),[32] China (n = 33),[33] and Brazil (n = 78)[16] to analyze polymorphism in the tandem Gln (Q) repeat region in block 6[13] or CB5 previously defined.[15] Frequency distribution of poly Q haplotypes was made for six countries, from which > 20 sequences were available. Sequences were aligned by using CLUSTAL W[34] implemented in MEGA software version 4[35] with manual corrections.

Sequence polymorphism was estimated by using the S, the number of polymorphic nucleotide sites; the number of singleton polymorphic sites; the number of polymorphic amino acid sites; the number of haplotypes and haplotype diversity *h*; $\theta\pi$, the observed average number of pairwise nucleotide difference per site; and θ_S, the standardized number of polymorphic nucleotide sites (S) per site in the sample expected under neutrality.[36] Tajima's D statistic was estimated for testing departure from neutrality with focus on allele frequency spectrum.[37] We used Tajima's D, which compares $\theta\pi$ and θ_S. Under neutrality, the value is expected to be 0; significantly positive values suggest recent population bottleneck or balancing selection, and negative values suggest population growth or directional selection. We also used Fu and Li's D* and F* tests to test for excess or lack of singleton nucleotides by comparing estimates of θ_S based on the number of singletons versus that derived from S (the D* index) or $\theta\pi$ (the F* index).[38] An excess of intermediate frequency polymorphisms or a lack of rare variants (including singleton nucleotides) results in positive values for D* and F*. All estimates were calculated by using DnaSP software version 4.10[39] and MEGA software.

The mean numbers of synonymous substitutions per synonymous site (dS) and nonsynonymous substitutions per nonsynonymous site (dN) were estimated by using the Nei and Gojobori method[40] with the Jukes and Cantor correction as implemented in MEGA. Standard error was determined by 1,000 bootstrap replications, and dN and dS were compared by using a Z-test of selection implemented in MEGA. If dN was significantly greater than dS, balancing selection appears to be acting. If dS was greater than dN, purifying selection is predicted. The McDonald-Kreitman test[41] was also used to assess a signature for selection, in which the ratio of nonsynonymous and synonymous substitutions was compared between polymorphic (within species) and fixed difference (between closely related species) by using DnaSP. Under neutrality these ratios will be similar, whereas an excess of intraspecific nonsynonymous polymorphisms is suggestive of balancing selection. Ten *msp1* sequences from *P. cynomolgi*,[12] a monkey malaria parasite closely related to *P. vivax*, were used for between-species comparison. Fisher's exact test was used to test for statistical significance. Microsatellite haplotypes (alelles) were determined by counting the number of microsatellite repeats.

RESULTS

Polymorphism of *pvmsp1* in isolates from Turkey. The PCR amplification of the whole *pvmsp1* was successful for 31 *P. vivax* isolates, and 30 full-length sequences were obtained: n = 20 from Siverek and n = 10 from Harran. One isolate from Harran had mixed genotype infection and was not used for further analysis. An alignment of the deduced amino acid sequence of *pvmsp1* from Turkey and other areas (n = 73) showed a number of sequence regions with insertions/deletions and/or tandem repeats of degenerative repeat units, which were scattered throughout the gene (Supplementary Figure 1). Because these sequence regions were not reliably aligned, they were excluded from further analysis, except for a region containing poly Q tandem repeats in block 6, which was used for repeat number polymorphism analysis (see below).

In the sequence region analyzed (4,728 basepairs), there were 543 and 299 polymorphic nucleotide sites in worldwide (n = 73) and Turkey samples, respectively (Table 1). The number of polymorphic sites in Turkey was less than two-thirds of that in Thailand and Brazil. In the 1,576 amino acid sites analyzed, amino acid substitutions were found in 280 and 162 sites in worldwide and Turkey samples, respectively (Supplementary Figure 1). Of the 162 amino acid changes, 8 were newly identified and not observed in other areas. These are N/S at amino acid position 60 (after the Sal-1 sequence), D/N at 931, K/N at 956, S/K at 958, G/T at 959, T/S at 968, P/A at 971, and D/E at 1706. Within Turkey, the number of polymorphic nucleotide and amino acid sites was somewhat lower in Harran than in Siverek. Of note, one of the eight changes that occurred in the C-terminal 19-kD region (D/E substitution at 1706) is a new report because, up to now, only the K/E change at 1709 was the sole polymorphism reported.[13,14] The K/E change was not detected in samples from Turkey.

The number of *pvmsp1* haplotypes was only three in Turkey with haplotype diversity (*h*) of 0.536, which is much lower than that in Thailand and Brazil (*h* = 0.974 and 0.944, respectively). Moreover, the three haplotypes found in Turkey were unique to this country. Both $\theta\pi$ and θ_S were also lower in Turkey (56% and 67%, respectively) than worldwide values (Table 1).

Polymorphism in *pvmsp1* poly Q region. The *pvmsp1* contains a highly polymorphic sequence region, which is characterized by the presence (Belem type) or absence (Sal-1 type) of tandem repeats of Gln (Q) residues in amino acid positions 726–748 in the Belem sequence (GenBank accession no. AF435594). Polymorphism of this region in isolates from Turkey was compared with that reported from 8 countries (Table 2). All samples from Turkey had Belem type and no Sal-1 type, whereas both types were present in samples from all other countries analyzed. Haplotype diversity in this sequence region was lowest in Turkey among nine countries for which frequency data are available. In Belem type, the number of Qs (10–31) and the frequency distribution of Q repeat haplotypes greatly varied among seven areas (Supplementary Figure 2). The frequency distribution in Turkey is clearly different from that in Iran and Azerbaijan, and the distribution in Iran and Afghanistan is apparently similar.

TABLE 1

Polymorphism and tests for departure from neutrality in *Plasmodium vivax* merozoite surface protein 1 from Turkey and other areas*

Country	No.	S	Ss	Sa	H	*h*	θπ	θS	Tajima D	Fu and Li D	Fu and Li F
Turkey	30	299	0	162	3	0.536 ± 0.077	0.02417 ± 0.00336	0.01596 ± 0.00092	1.7642 ($0.05 < P < 0.1$)	1.9747 ($P < 0.02$)	2.2545 ($P < 0.02$)
Siverek	20	299	0	162	3	0.426 ± 0.122	0.01720 ± 0.00512	0.01783 ± 0.00103	−0.2955 ($P > 0.1$)	1.8184 ($P < 0.02$)	1.3738 ($P > 0.1$)
Harran	10	224	0	119	2	0.533 ± 0.095	0.02610 ± 0.00448	0.01675 ± 0.00112	2.5404 ($P < 0.01$)	1.6706 ($P < 0.02$)	2.1353 ($P < 0.02$)
Thailand	20	495	24	259	16	0.974 ± 0.025	0.03955 ± 0.00309	0.02951 ± 0.00133	0.7668 ($P > 0.1$)	1.2955 ($0.05 < P < 0.1$)	1.3254 ($P > 0.1$)
Brazil	9	449	66	233	7	0.944 ± 0.070	0.04263 ± 0.00381	0.03494 ± 0.00165	0.7356 ($P > 0.1$)	1.0137 ($P > 0.1$)	1.0640 ($P > 0.1$)
Worldwide	73	543	26	280	36	0.918 ± 0.024	0.04326 ± 0.00115	0.02363 ± 0.00101	NA	NA	NA

* S = no. polymorphic nucleotide sites; Ss = no. singleton polymorphic nucleotide sites; Sa = no. polymorphic amino acid sites; H = No. haplotypes; *h* = haplotype diversity; θπ = observed average number of pairwise nucleotide difference per site ± SD; θS = standardized number of polymorphic nucleotide sites per site ± SD; NA = not available.

TABLE 2

Polymorphism of the *Plasmodium vivax* merozoite surface protwein 1 block 6 poly Q repeat region in isolates from Turkey and other areas*

Country	No.	H	*h*	No. Sal-1 type (%)	No. Belem type (%)	References
Turkey	30	3	0.536 ± 0.077	0 (0)	30 (100)	This study
Azerbaijan	36	7	0.614 ± 0.088	9 (25)	27 (75)	15
Iran	191	22	0.848 ± 0.016	80 (42)	111 (58)	41–43
Afghanistan	57	15	0.821 ± 0.039	31 (54)	26 (46)	44
Pakistan	33	9	0.873 ± 0.027	13 (39)	20 (61)	43
Myanmar	135	7	0.794 ± 0.016	111 (82)	24 (18)	20
Thailand	33	9	0.739 ± 0.057	28 (85)	5 (15)	23, 26
China	33	8	0.712 ± 0.059	17 (52)	16 (48)	47
Brazil	78	12	0.859 ± 0.016	25 (32)	53 (68)	3, 26

* H* = no. haplotypes; *h** = haplotype diversity ± SD.

Departure from neutrality. Three tests were applied to detect departure from neutrality in *pvmsp1*. The Z-test for a difference between dN and dS showed significantly higher dS than dN (Figure 3) in Turkey, Thailand, and Brazil. When the whole sequence (4,728 basepairs) was divided into three regions (the N-terminal, central, and C-terminal regions), dS was significantly higher than dN in the N-terminal and central regions for all three countries, whereas in the C-terminal 42-kD region, dN was significantly higher than dS in Thailand, Brazil, and India but not in Turkey. In the 19-kD C-terminal region, dN > dS was not significant because there were only two substitutions in this region (Supplementary Figure 1).

Using Tajima's D statistics and Fu and Li's statistics (D* test and F* test), we found that Tajima's D value was weakly (but not significantly) positive only in Turkey. Fu and Li's D* and F* values were significantly positive for Turkey but not for Thailand and Brazil. Within Turkey, samples from Harran yielded significantly positive values for the three statistics. If we consider recent rapid reduction in the incidence of malaria in the study areas (Figure 2), these results suggest a recent bottleneck in *P. vivax* populations in Turkey.

The McDonald and Kreitman test showed significantly higher intraspecific nonsynonymous substitutions than synonymous substitutions in parasite populations from Turkey, Thailand, and Brazil (Table 3), suggesting balancing selection acting on *pvmsp1* in all areas. No signature of balancing selection was evident for *P. cynomolgi*, as reported.[6] Sequence regions showing balancing selection in *pvmsp1* were the central and C-terminal 42-kD regions. A population from India also showed significantly higher intraspecific nonsynonymous substitutions than synonymous substitutions in the 42-kD region. Although high intraspecific nonsynonymous substitutions over synonymous substitutions in a locus is also observed when constraints are relaxed,[42] no singleton alleles were found in samples from Turkey (Table 1), making the occurrence of relaxed constraints in *pvmsp1* unlikely.

Polymorphism in microsatellites. The number of microsatellite repeats (alleles) was only three in four loci examined, with $h = 0.55$ (range = 0.476–0.626) (Table 4). These values are considerably lower than *h* values from other areas: 0.72–0.79 in India, Laos, Thailand, and Colombia[28]; 0.86 in Vietnam[43]; 0.79 in Sri Lanka[27]; and 0.80 in Brazil.[44]

DISCUSSION

The present analysis of *pvmsp1* polymorphism showed a remarkably lower diversity in *P. vivax* populations in Turkey

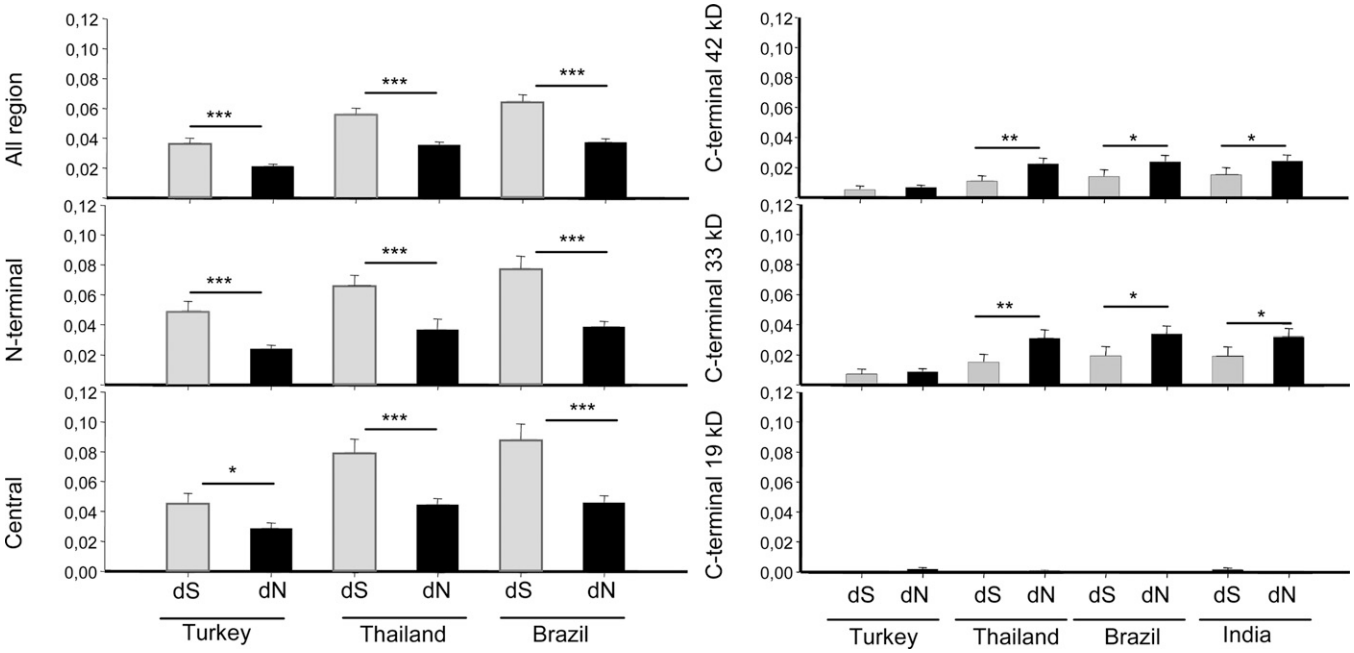

FIGURE 3. Rate of synonymous (dS) and nonsynonymous (dN) substitutions per synonymous and nonsynonymous sites in the *Plasmodium vivax* merozoite surface protein gene (*pvmsp1*) from Turkey, Thailand, and Brazil. The *pvmsp1* sequence was separated into three regions: the N-terminal, central, and C-terminal regions. The C-terminal 42-kD polypeptide region was further divided into the 33-kD and 19-kD regions. $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$.

than in populations in Thailand and Brazil. Nucleotide diversity was 56–67% of worldwide samples. Nonetheless, there were eight new single nucleotide polymorphisms identified that were unique to Turkey isolates. This finding indicates that additional *pvmsp1* sequences from various areas are still likely to be required to fully document polymorphism of the gene. Among the eight single nucleotide polymorphisms, D/E substitutions at 1706 are notable because they reside in the C-terminal 19-kD polypeptide, a candidate vaccine molecule. Given that the amino acid change is not radical, i.e., D and E are acidic residues, it may not cause a dramatic effect on the function of the polypeptide during erythrocyte invasion by the merozoite. However, the substitution may potentially lead to a change of antibody binding because the position is surface exposed.[45]

The observed low diversity of *pvmsp1* in Turkey has implications regarding acquisition of immunity against *P. vivax* malaria. It is believed that repeated infections are required for persons in malaria-endemic areas to effectively mount antimalaria protective immunity. This belief largely stems from

TABLE 3
McDonald and Kreitman test results for *Plasmodium vivax* merozoite surface protein 1 from Turkey and other areas*

			WSPD			WSPD			WSPD			WSPD	
			P. vivax	*P. cynomolgi*		*P. vivax*	*P. cynomolgi*		*P. vivax*	*P. cynomolgi*		*P. vivax*	*P. cynomolgi*
pvmsp1 region	Substitution type	BSFD	Turkey		BSFD	Thailand		BSFD	Brazil		BSFD	India	
Whole gene	Synonymous	173	69	196	155	105	195	156	105	195			
	Nonsynonymous	176	118	191	153	182	182	162	163	184	NA	NA	NA
			$P = 0.005$	$P = 0.77$		$P = 0.007$	$P = 0.72$		$P = 0.017$	$P = 0.53$			
5′ region	Synonymous	62	47	80	51	65	79	52	67	79			
1,851 bp	Nonsynonymous	80	76	97	65	110	88	72	98	90	NA	NA	NA
			$P = 0.369$	$P = 0.784$		$P = 0.244$	$P = 0.579$		$P = 0.820$	$P = 0.413$			
Central	Synonymous	58	20	68	51	37	68	52	34	68			
1,227 bp	Nonsynonymous	39	28	48	33	51	48	34	43	48	NA	NA	NA
			$P = 0.039$	$P = 0.862$		$P = 0.014$	$P = 0.766$		$P = 0.037$	$P = 0.719$			
3′ region	Synonymous	53	2	48	53	3	48	52	4	48	52	7	47
(42 kD)	Nonsynonymous	57	14	46	55	21	46	56	22	46	55	25	46
1,098 bp			$P = 0.017$	$P = 0.682$		$P = 0.002$	$P = 0.778$		$P = 0.005$	$P = 0.679$		$P = 0.013$	$P = 0.784$
33 kD	Synonymous	43	2	39	43	3	39	42	4	39	42	6	39
789 bp	Nonsynonymous	46	13	37	45	20	37	45	22	37	45	25	37
			$P = 0.012$	$P = 0.701$		$P = 0.004$	$P = 0.754$		$P = 0.006$	$P = 0.699$		$P = 0.009$	$P = 0.698$
19 kD	Synonymous	10	0	9	10	0	9	10	0	9	10	1	8
309 bp	Nonsynonymous	11	1	9	10	1	9	11	0	9	10	0	9
			$P = 0.454$	$P = 0.863$		$P = 1.0$	$P = 1.0$		NA	$P = 0.863$		$P = 1.0$	$P = 0.879$

**pvmsp1* = *P. vivax* merozoite surface protein 1; BSPD = between-species fixed difference; WSPD = within-species polymorphic difference; NA = not available; bp = basepairs. *P* values < 0.05 are shaded in grey.

TABLE 4
Microsatellite haplotype diversity of *Plasmodium vivax* population in Turkey

Microsatellite locus	No.	Repeat unit	No. repeat types	Haplotype diversity (*h*)
MS8	30	CA(C/G)	3	0.476 ± 0.091
MS9	29	AGG	3	0.626 ± 0.048
MS15	29	CTT	3	0.488 ± 0.091
3.502	26	ATGAACGG	3	0.594 ± 0.073
				Mean ± SD = 0.546 ± 0.075

epidemiologic observations from areas highly endemic for *P. falciparum* malaria in sub-Sahara Africa, where a number of distinctive genotypes are circulating and mixed genotype infections are commonly seen.[46] Strain-specific protective immunity (SSPI) or allele-linked immunity is strongly suggested to be involved in the slow acquisition of protective immunity against *P. falciparum* malaria.[47] In a rodent malaria model, SSPI has been shown to exist,[48] and parasite MSP-1 has strongly been suggested to be the principal candidate molecule for the control of SSPI against *P. chabaudi chabaudi* malaria.[49,50] If strain-specific immunity is true for *P. vivax*, it is predicted that acquisition of immunity may be faster in Turkey than in other malaria-endemic areas, such as Thailand[13,32] and Brazil,[16] where *pvmsp1* diversity is relatively high. In Brazil, antibodies against polymorphic regions of PvMSP-1 are slow to develop, compared with the conserved C-terminal 19-kD polypeptide, which suggests that repeated infections are required to elicit antibody responses to variable sequence regions.[16] In Sri Lanka, antibody prevalence was higher to the 33-kD polymorphic region than to the 19-kD region.[51] Cohort studies to correlate protective immunity with antibody responses against PvMSP-1 (polymorphic regions and conserved regions, including the C-terminal 19-kD polypeptide) would be required to infer a role of *pvmsp1* polymorphism in immune evasion.

In general, the level of genetic diversity of the parasite may be determined by several variables such as population evolutionary/demographic history, effective population size, gene flow between neighboring populations, and natural selection. In a population that has an old origin and large effective population size, a higher genetic diversity would be expected than a population with a recent origin and small effective population size. Microsatellite diversity was lower in Turkey than in other areas, which suggested a relatively small effective population size in the parasite population studied. In Turkey, human movements from and to neighboring countries is strictly limited, and parasite gene flow accompanied by human movements would be too low to cause introduction of parasite variants not prevalent in Turkey or increase genetic diversity. The frequency distribution pattern of poly Q repeat haplotypes (Supplementary Figure 2) was distinctive between Turkey and neighboring countries (Azerbaijan and Iran), and the distribution pattern was apparently similar between Iran and Afghanistan. Contribution of human movements to increasing genetic diversity has been observed between Iran, Afghanistan and Pakistan.[23,30] In this study, Fu and Li's D* and F* tests showed significantly positive values for populations from Turkey, which suggested a recent parasite population bottleneck. Consistent with this finding is the dramatic decrease in annual incidence of malaria in Sanliurfa, where there were two waves of rapid reductions in the early 1980s and after 1999 until the present time (Figure 2).

Additionally, the present population genetic study indicates that a signature of balancing selection on *pvmsp1* appears differently among *P. vivax* populations. In Thailand, Brazil, and India, an excess of dN over dS was observed in the C-terminal 42-kD polypeptide; this finding was not detected in Turkey. The McDonald-Kreitman test detected balancing selection for the central and C-terminal polypeptides in Turkey, Thailand, and Brazil (and in India for the 42-kD fragment). A potential population bottleneck in the *P. vivax* populations in the study area after a rapid reduction of *P. vivax* cases caused by recent extensive malaria interventions may be associated with the failure of detecting an excess of dN over dS in Turkey.

In conclusion, the present study demonstrates low diversity of *pvmsp1* in *P. vivax* isolates from Turkey. The low antigen diversity should be informative for gaining insights of acquired immunity against *P. vivax* malaria. A population bottleneck of *P. vivax* was inferred, which was probably caused by recent malaria intervention efforts in the study area. A signature of balancing selection on *pvmsp1* was obscured in a parasite population that had experienced a bottleneck, which suggested geographic differences in balancing selection in an antigen gene of *P. vivax*.

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